Biochemistry and Biophysics Reports 2 (2015) 137–142

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Liposomal delivery of a phosphodiesterase 3 inhibitor rescues low oxygen-induced ATP release from erythrocytes of humans with type 2 diabetes

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ARTICLE INFO

Article history:
Received 15 February 2015
Received in revised form
20 May 2015
Accepted 27 May 2015
Available online 30 May 2015

Keywords:
Drug delivery
Liposomes
Erythrocytes
Diabetes
Phosphodiesterase inhibitors

ABSTRACT

ATP release from erythrocytes in response to low oxygen tension requires an increase in cAMP, the level of which is regulated by phosphodiesterase 3 (PDE3). Such release is defective in erythrocytes of humans with type 2 diabetes (DM2). This study tested a hypothesis that direct delivery of the clinically useful PDE3 inhibitor, cilostazol, to erythrocytes of humans with type 2 diabetes using liposomes would restore low-oxygen tension-induced ATP release. Cilostazol was incorporated into liposomes prepared from dimyristoylphosphatidylcholine (DMPC). Liposome-delivery of cilostazol restored ATP release from DM2 erythrocytes to levels which were not different from that released from non-cilostazol treated healthy erythrocytes under the same conditions. There were no observed adverse effects of the liposomes on either healthy or DM2 erythrocytes. The directed liposomal delivery of PDE inhibitors to erythrocytes may help prevent or slow the development of peripheral vascular disease in individuals with DM2 by restoring an important physiological controller of microvascular perfusion while minimizing side effects associated with systemic delivery of some of these inhibitors.

1. Introduction

Erythrocytes, in addition to carrying and delivering oxygen (O_2), have been shown to play an active role in the distribution of local microvascular perfusion via their ability to release the potent vasodilator, ATP. Several physiological stimuli for erythrocyte ATP release have been identified including exposure of the cells to prostacyclin (PGI_2) analogs and low O_2 tension. Indeed, low O_2-induced release of ATP has been suggested to be an important regulator of perfusion distribution and oxygen supply in skeletal muscle [1,2]. As such, a defect in this pathway would be expected to result in alterations in normal physiological function. Erythrocytes obtained from humans with type 2 diabetes (DM2), a disease associated with an increased incidence of peripheral vascular disease, fail to release ATP in response to low O_2 [3–5]. It is reasonable to hypothesize that restoration of low O_2-induced ATP release from DM2 erythrocytes could be important for the prevention and treatment of the claudication and delayed wound healing associated with this disease.

The signaling pathways for ATP release in response to exposure to PGI_2 or low O_2 have been elucidated and, although the pathways differ in several ways, in both pathways, ATP release requires increases in intracellular cyclic adenosine monophosphate (cAMP). In all cells, cAMP levels are determined by the rate of synthesis by adenylyl cyclases and degradation by phosphodiesterases (PDEs) [6]. In the PGI_2 and low O_2 signaling pathways in human erythrocytes, the phosphodiesterase responsible for degradation of cAMP is a pathway specific isoform of PDE3 (Fig. 1) [7].

If increases in cAMP are required for ATP release from erythrocytes, inhibition of the PDE that degrades that cyclic nucleotide could rescue low O_2-induced ATP release from DM2 erythrocytes [3]. Indeed, it was shown that incubation of DM2 erythrocytes with a selective PDE3 inhibitor (cilostazol) restored ATP release when these cells were exposed to low O_2 [3]. The importance of the restoration of this physiological response to low O_2 is illustrated by the finding that cilostazol also restored the ability of DM2 erythrocytes to stimulate dilation of isolated skeletal muscle arterioles exposed to reduced extraluminal O_2, a model of increased tissue O_2 need [3].

Although such studies have provided strong support for the use

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http://dx.doi.org/10.1016/j.bbrep.2015.05.011

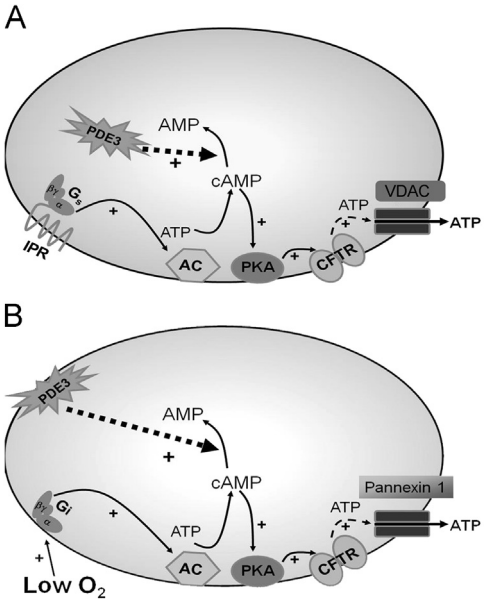

Fig. 1. Signaling pathways for ATP release from erythrocytes in response to prostacyclin analogs (A) and low O_2 tension (B). Abbreviations: AC: adenylyl cyclase; AMP: adenosine monophosphate; ATP: adenosine 5′ triphosphate; cAMP: 3′,5′-cyclic adenosine monophosphate; CFTR: cystic fibrosis transmembrane conductance regulator; Gi and Gs: the heterotrimeric G proteins, Gi and Gs, respectively IPR: prostacyclin receptor; PDE3: phosphodiesterase 3; PKA: protein kinase A; VDAC: voltage-dependent anion channel; (+): activation.

of PDE3 inhibitors in DM2, oral or intravenous administration of these drugs has been associated with the unwanted side-effects of cardiac arrhythmias and worsening of heart failure [8]. Thus, the use of PDE3 inhibitors in DM2 has not been widely adopted. Targeted delivery of PDE3 inhibitors to erythrocytes in DM2 patients may alleviate the side effects while providing the benefits of restoration of ATP release in response to low oxygen. Here we report the results of feasibility studies undertaken to develop liposomes that could deliver PDE3 inhibitors directly to erythrocytes. This approach could permit the use of these drugs in the treatment of DM2-associated vascular disease while avoiding unwanted cardiac side effects.

Liposomes have been used to encapsulate a broad range of water-insoluble (lipophilic) or hydrophilic drugs [9–12]. Targeting specific tissues using liposomes has been challenging, in part because liposomes interact extensively with components of blood, including erythrocytes [13–16]. However, this challenge for liposome-based delivery vehicles to tissues is a distinct advantage for their delivery to erythrocytes. Here we show that dimyristoylphosphatidylcholine (DMPC) liposomes formulated with cilostazol can be taken up by erythrocytes and that liposome-delivered cilostazol increases the release of ATP from DM2 erythrocytes.

2. Materials and methods

2.1. Preparation of liposomes

Unilamellar liposomes were prepared by the extrusion method [17]. DMPC (20 mg) was added to a 2 mM (0.71 mg) solution of cilostazol in a methanol/chloroform (50/50) mixture. The solvent was evaporated using a stream of purified argon to form a lipid/drug film on the walls of a culture tube. The lipid film was further dried under vacuum for 60 min to remove traces of chloroform and methanol. The dried film was hydrated with 1 mL of buffer solution (in mM: 4.7 KCl, 2.0 $CaCl_2$, 140.5 NaCl, 1.2 $MgSO_4$, 21.4 $NaHCO_3$, pH 7.4 at 37 °C) giving a dispersion of multilamellar vesicles. The solution was then extruded through a polycarbonate membrane with 0.1 μm pores using an Avanti Mini-Extruder (Avanti Polar Lipids). Control liposome samples were prepared following the same procedure but without cilostazol.

2.2. DLS analysis

Hydrodynamic diameter and polydispersity index (PDI) measurements were performed on a Malvern Nano-ZS Zetasizer (Malvern Instruments Ltd., Worcestershire, U.K.). The Helium-Neon laser, 4 mW, operated at 633 nm, with the scatter angle fixed at 173°, and the temperature at 25 °C. 80 μL samples were placed into disposable cuvettes without dilution (70 μL, 8.5 mm center height Brand UV-Cuvette micro). Each data point was an average of 10 scans.

2.3. HPLC analysis of cilostazol in liposomes

An aliquot of liposome solution (50 μL) was diluted with methanol (approximately 1:20) and the solution was analyzed using an HPLC equipped with a UV detector and a reverse-phase column (C18, 3.9×150 mm^2) with methanol as the mobile phase. When the flow rate was set to 0.5 mL/min, the retention time for cilostazol was approximately 1.5 min. Samples were collected during preparation of liposomes as follows: Sample 1: a stock solution of cilostazol in methanol (reference sample); Sample 2: a lipid mixture with cilostazol in methanol; Sample 3: liposomes with cilostazol immediately after hydration and extrusion; Sample 4: liposomes with cilostazol passed through Sephadex G50 column. All samples showed similar results. The finding that the results from the first 3 steps were identical within experimental error was interpreted to mean that all of the cilostazol was incorporated into liposomes. The result for Sample 4 equaled 87–95% of the amount measured in Sample 3. No drug was observed in the eluent after the liposomes were collected.

2.4. LCMS determination of liposomes in erythrocytes

Phospholipids were extracted from the erythrocytes using the method of Bligh and Dyer [18]. The chloroform–methanol layer was removed, dried and resuspended in 1.5-mL of methanol. LCMS analysis was used to detect the presence of cilostazol and lipids from liposomes in the RBC membranes after incubation.

2.5. Stability of liposomes loaded with cilostazol

Samples of DMPC liposomes loaded with cilostazol were prepared as described above and incubated at 4 °C. Every 24 h, an aliquot was taken for the DLS analysis of average size and size distribution of liposomes. DLS measurements were performed as described above. Stability of samples was monitored for one week.

2.6. Isolation of human erythrocytes

Whole blood was obtained from healthy volunteers ($n=15$) and patients with type 2 diabetes (DM2, $n=6$) by venipuncture and collected into a syringe containing 500 units of heparin. The blood was centrifuged at 500 g for 10 min at 4 °C. The plasma, buffy coat, and less than 1% of the erythrocyte mass were removed by aspiration and discarded. The packed erythrocytes were resuspended and washed three times in a physiological buffer containing (in mM) 21.0 tris(hydroxymethyl) aminomethane, 4.7 KCl, 2.0 $CaCl_2$, 140.5 NaCl, 1.2 $MgSO_4$, and 5.5 glucose with 0.5% bovine serum albumin fraction V, final pH 7.4. Erythrocytes were prepared on the day of use.

2.7. Measurement of ATP

Washed erythrocytes were diluted to a 20% hematocrit in the physiological buffer. ATP was measured by the luciferin–luciferase technique [19]. Briefly, a 200 μL sample of the erythrocyte suspension (0.04% hematocrit) was injected into a cuvette containing 100 μL of 10 mg/mL crude firefly lantern extract (Sigma, St. Louis, MO) and 100 μL of 0.5 mg/mL D-luciferin (Research Products International, Mt. Prospect, IL). The light emitted was detected using a luminometer (Turner Designs, Sunnyvale, CA). An ATP standard curve was generated on the day of each study. ATP levels are reported as nanomoles per 4×10^8 erythrocytes.

2.8. Measurement of free hemoglobin

To exclude the presence of significant hemolysis in studies where the release of ATP was measured, samples were centrifuged at 500*g* for 10 min at 4 °C. The presence of free hemoglobin in the supernatant was determined by light absorption at a wavelength 405 nm [20].

2.9. Determination of the effect of the prostacyclin analog, UT-15C, on ATP release from healthy human erythrocytes in the presence of blank liposomes or liposomes containing cilostazol

Washed erythrocytes from healthy humans were diluted to a 20% hematocrit in the physiological buffer described above. Erythrocytes were incubated with either blank liposomes or liposomes containing cilostazol (Sigma, St. Louis, MO) for 30 min prior to the addition of UT-15C (100 nM, United Therapeutics, Research Triangle Park, NJ). ATP was measured prior to and at 5, 10, and 15 min after addition of UT-15C.

2.10. Determination of ATP release from erythrocytes in response to exposure to reduced O_2 tension in the presence of blank liposomes or liposomes containing cilostazol

Washed erythrocytes of healthy humans and humans with DM2 were diluted to a 20% hematocrit in a Ringers buffer containing bicarbonate (in mM; 4.7 KCl, 2.0 $CaCl_2$, 140.5 NaCl, 1.2 $MgSO_4$, 5.5 glucose, 21.4 $NaHCO_3$, 0.5% BSA, pH 7.4 at 37 °C). Cells were equilibrated for 30 min with a gas mixture containing 15% O_2, 6% CO_2, balance N_2 ($pH = 7.41 \pm 0.03$, $pCO_2 = 36 \pm 2$ mmHg and $pO_2 = 107 \pm 5$ mmHg; normoxia) in a thin-film tonometer (model 237; Instrumentation Laboratory, Bedford, MA) in the presence of blank liposomes or liposomes containing cilostazol for 20 min. The gas mixture was then changed to one containing 0% O_2, 6% CO_2, balance N_2 ($pH = 7.42 \pm 0.02$, $pCO_2 = 38 \pm 2$ mmHg and $pO_2 = 10 \pm 1$ mmHg; low O_2 tension). The concentration of ATP released from erythrocytes was determined during normoxia and following a 10 min exposure of erythrocytes to low pO_2. The pH, pO_2, and pCO_2 were determined during exposure to each gas mixture using a blood gas analyzer (model pHOx; Nova Biomedical, Waltham, MA).

2.11. Institutional approval

Informed consent was obtained from all subjects. The protocol for blood removal was approved by the Institutional Review Board of Saint Louis University. All record keeping was in strict compliance with the Health Insurance Portability and Accountability Act (HIPAA) regulations.

2.12. Statistical analysis

Statistical significance among groups was determined using an analysis of variance (ANOVA). In the event that the *F* ratio indicated that a change had occurred, a Fisher's LSD test was performed to identify individual differences. Results are reported as mean ± the standard error (SE).

3. Results and discussion

3.1. Preparation and characterization of cilostazol-loaded liposomes

Liposomes were prepared by the hydration of dried lipid film with or without cilostazol followed by extrusion using a previously published procedure [17]. Several different phospholipids were used for this study, dioleoylphosphatidylcholine (DOPC), dimiristoylphosphatidylcholine (DMPC), dilauroylphosphatidylcholine (DLPC), and dipalmitoylphosphatidylcholine (DPPC). Throughout each experiment, each sample was subjected to dynamic light scattering (DLS) analysis, providing a value for the average size and the polydispersity index (PDI) of the liposomes in the sample. DLS data (Fig. 2A) show the formation of liposomes with identical sizes with and without cilostazol with low PDI. Fig. 2A shows the data for DMPC; similar results were obtained with all other lipids.

The amount of cilostazol associated with liposomes was determined by high-performance liquid chromatography (HPLC) following a previously published protocol [21]. During the preparation of liposomes with cilostazol, samples for the HPLC analysis were collected throughout the preparation steps as mentioned in the experimental section. Separation in a size-exclusion column was done to confirm that cilostazol was associated with the liposomes. The total quantity of cilostazol found in Samples 1–3 was the same. Sample 4 (liposomes with cilostazol passed through a size-exclusion column) showed 87–95% of the amount found in Sample 3 (liposomes with cilostazol after extrusion and before size-exclusion column). Considering the high likelihood of small sample loss during collection from a size-exclusion column, this finding indicates that all or nearly all cilostazol used for the preparation was associated with liposomes. If cilostazol were located predominantly in the aqueous phase, it would have been separated out in the size exclusion separation. Liposomes with the diameter of approximately 100 nm (Fig. 2A) elute with the void volume of the column and are readily separated from small molecules. The inner aqueous volume in this preparation is below 5% of the total sample volume; therefore, if cilostazol were located predominantly in the aqueous phase, preparation 4 would have shown much lower amounts of cilostazol. In further control measurements, all subsequent fractions eluted from the size exclusion column collected after the liposome fraction did not show any cilostazol. To demonstrate that cilostazol was effectively delivered to erythrocytes using liposome vehicles, ATP release in response to receptor-mediated activation of the prostacyclin receptor (IPR) by the prostacyclin analog, UT-15C, was determined in the absence and presence of cilosatzol-loaded liposomes. The concentration of UT-15C that was used (100 nM) is insufficient to stimulate ATP release from healthy human erythrocytes in the absence of an inhibitor of PDE3.

In addition to measuring UT-15C-induced ATP release, erythrocytes were examined under a microscope to evaluate any potential morphological changes. In initial experiments, UT-15C was unable to stimulate ATP release in the presence of liposomes prepared from DOPC. Considering that cilostazol was associated with these liposomes, we concluded that DOPC liposomes were not an effective vehicle for cilostazol delivery, most likely due to ineffective or slow interactions with the membrane of erythrocytes. To facilitate fusion of liposomes with erythrocytes, we tested two saturated phospholipids having the phase transition temperature below physiological range, DMPC (24 °C) and DLPC

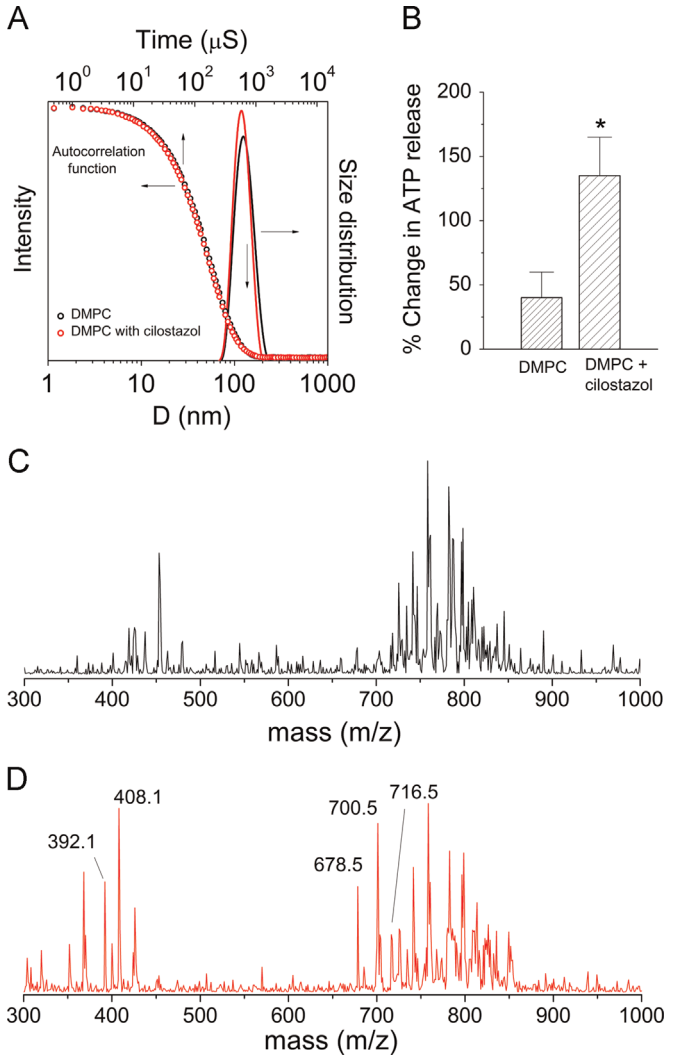

Fig. 2. (A) Size distribution (solid lines) and autocorrelation function (open circles) of liposomes with and without cilostazol determined by DLS in aqueous solution. (B) Percent change in ATP release in response to the prostacyclin analog UT-15C (100 nM) for healthy human erythrocytes ($n=8$) in the presence of empty DMPC liposomes and DMPC liposomes containing cilostazol. Values are means ± SE *different from respective control, $p<0.05$. (C and D) LCMS spectra of control erythrocytes (C) and liposome-treated erythrocytes (D). These studies demonstrate that liposomes are a viable vehicle for the delivery of PDE3 inhibitors to human erythrocytes and that the lipid composition of the liposomes plays an important role in the successful delivery of cilostazol. In our studies, DMPC liposomes proved to be the most effective in the delivery of cilostazol to erythrocytes.

(−2 °C). In control experiments we found that DLPC caused substantial changes in morphology of erythrocytes upon brief incubation. In contrast, DMPC liposomes showed no adverse effect on erythrocyte morphology. Importantly, incubation of healthy human erythrocytes with DMPC containing cilostazol significantly increased UT-15C-induced ATP release (Fig. 2B).

We also tested liposomes made of DPPC that had longer hydrocarbon chains and a higher phase transition temperature (41 °C). While we saw no adverse effect of these liposomes on erythrocyte morphology, we did not observe increases in ATP release from erythrocytes in response to low O_2 tension suggesting that cilostazol was not delivered effectively to by DPPC liposomes. These findings are in agreement with a previously published report on delivery of bilayer-associated pharmaceuticals to erythrocytes [22].

LCMS analysis was used to detect the presence of cilostazol and lipids from liposomes in the membranes of erythrocytes after incubation. In these experiments, erythrocytes were incubated with cilostazol-loaded liposomes then washed to remove free liposomes and any molecules in the buffer solution. Erythrocytes were subjected to hypotonic lysis. Hemoglobin and other cytoplasmic components were discarded after centrifugation. Lipids and other hydrophobic components of the pellet were extracted with chloroform. The extracts were dried to measure the amount of dry residue, and the solid components were dissolved in methanol and analyzed by mass spectrometry. Fig. 2C shows the mass spectrum of erythrocytes before incubation with cilostazol-loaded liposomes.

Lipids of an erythrocyte membrane are typically detected in the *m/z* region between 600 and 860. The region below $m/z=600$ is dominated by metabolites of lipids, primarily 1-lysophosphatidylcholines (lyso-PC). An erythrocyte membrane consists of various lipids that differ in head group type (i.e., phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine) and in their acyl chain length and saturation level [23,24]. These lipids give rise to a complex signature in the mass spectrum (Fig. 2C). Lipids from liposomes, however, can easily be detected and identified in erythrocyte membranes based on characteristic *m/z* values. In electrospray ionization, three different quasi-molecular ions are created from one molecule due to association of a molecule with H^+, Na^+, or K^+, resulting in three different characteristic peaks in the mass spectrum. DMPC peaks appear at *m/z* 678.5, 700.5, and 716.5, and cilostazol peaks are visible at *m/z* 392.1 and 408.1 (Fig. 2D). Stoll et al. [22] showed that the short acyl chain length lipids have a greater affinity to either incorporate into the erythrocyte membranes or stick to it compared to the long acyl chain length lipids. The presence of both DMPC and cilostazol molecules in the erythrocytes unambiguously confirms successful delivery of cilostazol to erythrocytes with the help of liposomes.

We examined the stability of liposomes loaded with cilostazol. DLS data (Fig. 3) show no change in the average size and size distribution of liposomes during one week of storage at 4 °C. Fusion of liposomes, a major reason for their instability, would

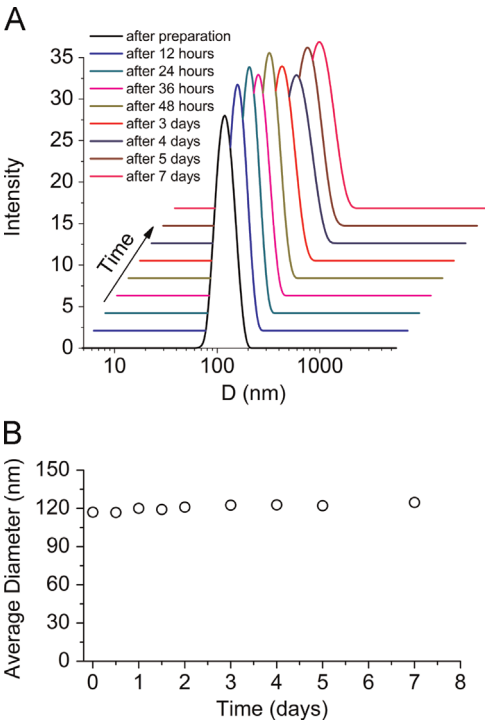

Fig. 3. Stability of DMPC liposomes loaded with cilostazol evaluated by DLS measurements. (A) Stacked DLS data acquired daily for one week and (B) average sizes of liposomes during the one-week period.

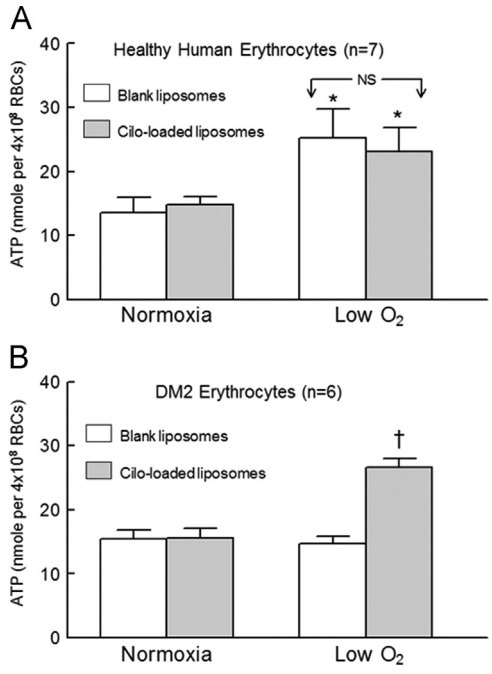

Fig. 4. ATP release from healthy human ($n=7$) (A) and DM2 erythrocytes ($n=6$) (B) incubated with blank liposomes or liposomes containing cilostazol during normoxia and following exposure to reduced oxygen tension. Values are means ± SE. *; different from respective normoxia, $p<0.05$: †different from respective normoxia, $p<0.01$: NS; not different.

manifest itself in the increase of the average size and broadening of the size distribution. Lack of change in these parameters (Fig. 3) indicates the stability of liposomes. Experiments on the delivery of cilostazol to erythrocytes described below were performed at different times following the preparation of the liposomes loaded with cilostazol, between several hours and one week with no measurable difference. The stability of cilostazol-loaded DMPC liposomes is similar to the stability of blank DMPC liposomes reported previously [21].

3.2. Impact of liposome-delivered cilostazol on ATP release from healthy Human and DM2 erythrocytes

Previous studies demonstrated that incubation of DM2 erythrocytes with cilostazol restores ATP release in response to low O_2 tension [1]. To evaluate the ability of cilostazol-containing liposomes to similarly rescue low O_2-induced ATP release from DM2 erythrocytes, cells from both healthy humans and humans with DM2 were incubated with liposomes prepared from DMPC with or without cilostazol.

First, in a set of control experiments, we determined that healthy erythrocytes are not adversely affected by DMPC liposomes and that they respond to liposome-delivered cilostazol. When incubated with liposomes that did not contain cilostazol, healthy human erythrocytes released ATP when exposed to reduced O_2 tension (Fig. 4A). The release of ATP was the same as previously observed for healthy erythrocytes in the absence of any treatment. Importantly, these cells also released ATP in response to this stimulus when incubated with liposomes containing cilostazol. These findings illustrate that DMPC liposomes loaded with the PDE3 inhibitor had no adverse effect on healthy human erythrocytes. As mentioned above, the effect of liposomes on erythrocytes is lipid-specific.

DM2 erythrocytes did not release ATP when exposed to reduced O_2 in the presence of blank liposomes (Fig. 4B). This outcome is identical to the normal behavior of DM2 erythrocytes in reduced O_2 environment [1]. When incubated with liposomes containing cilostazol, this stimulus resulted in ATP release that was not different from the amount released from healthy human erythrocytes under the same conditions (Fig. 4A and B). This finding is similar to previously published results demonstrating that incubation of DM2 erythrocytes with cilostazol in solution. That study demonstrated that incubation of DM2 erythrocyte with cilostazol (100 μM) resulted in a 105 ± 45% increases in ATP release when the cells were exposed to low oxygen tension [3]. Here we show that exposure of DM2 erythrocytes to liposomes loaded with cilostazol also rescues low oxygen-induced ATP release and that the increases is 107 ± 20% ($p<0.05$, Fig. 4). Thus, the results presented here establish that (1) liposome-mediated delivery of PDE3 inhibitors to erythrocytes of humans with DM2 restores the physiological response to exposure to low O_2 and (2) the magnitude of the rescue is similar to that observed when cilostazol is incubated directly with these cells [3]. Combined with experiments showing retention of cilostazol in liposomes, these studies confirm successful liposomal delivery of cilostazol and demonstrate the feasibility of achieving targeted delivery of PDE inhibitors to erythrocytes.

In summary, we demonstrated the successful delivery of cilostazol, a PDE3 inhibitor, to erythrocytes, using liposomes as vehicles. The efficiency of the delivery of cilostazol to erythrocytes is sensitive to the liposome formulation. Using DMPC liposomes, we were able to achieve quantitative delivery of cilostazol. Importantly, liposome-delivered cilostazol rescued the ability of erythrocytes of humans with DM2 to release ATP in response to low O_2. Targeting of erythrocytes for the delivery of PDE3 inhibitors could have significant translational importance in the treatment of DM2.

Traditionally, formulations of liposomes for drug delivery were designed to prolong the circulation time of drugs and to minimize their interactions with blood components. Here we exploited the potential for interaction of liposomes with erythrocytes to our advantage. Systemic effects of PDE3 inhibitors have limited their usefulness in DM2 [8]. The targeted delivery of these drugs to erythrocytes would minimize their unwanted side effects and represents a novel approach to the treatment of the vascular disease and delayed wound healing associated with DM2.

Acknowledgments

This work was supported by the National Science Foundation (CHE-1316680 and CHE-1522525), the American Diabetes Association, and the Saint Louis University Presidential Research Fund. We thank United Therapeutics for the gift of UT-15C.We thank J.L. Sprague for inspiration.

Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at doi:10.1016/j.bbrep.2015.05.011

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